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Effectiveness in Enhancing Oil Recovery through Combination of Biosurfactant and Lipases Bacteria

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ABSTRACT

Oil pollution accidents recently became phenomenon and caused accidental contamination of ecosystem. It originated from leaking pipes, transportation accidents, and damage oil storage tanks that contaminated both soil and groundwater. One of the solution was oil recovery process using sand pack column with combination of biosurfactant (*Bacillus subtilis* 3Kp, *Pseudomonas putida* T1-8, *Micrococcus* sp. L II 61 and *Acinetobacter* sp. P2(1)) and lipases (*Actinobacillus* sp., *Micrococcus* sp. L II). S*and-pack column* model designed to simulate oil recovery operations and evaluate the mobilization of residual oil by combined biosurfactants and lipases. This study was an experimental study through four replications. Treatment were consists of eight combinations; *Acinetobacter* sp. P2 (1) - *Actinobacillus* sp., *Bacillus subtilis* 3Kp - *Actinobacillus* sp., *Pseudomonas putida* T1-8 - *Actinobacillus* sp., *Micrococcus* sp. L II 61 - *Actinobacillus* sp., *Acinetobacter* sp. P2 (1) - *Micrococcus* sp. L II 61, *Bacillus subtilis* 3Kp - *Micrococcus* sp. L II 61, *Pseudomonas putida* T1-8 - *Micrococcus* sp. L II 61 and biosurfactant and lipase *Micrococcus* sp. L II 61 with positive control synthetic surfactant (Tween-20). The results of treatment were extracted using n-hexane. Effectiveness of oil recovery by biosurfactants and lipases combination analyzed using One-way ANOVA test. These eight combinations effectively mobilize of entrapped oil as indicated by high percentage of oil recovered compared to the synthetic surfactants (Tween-20). Thus, the eight types of combination were capable to replace synthetic surfactants in oil recovery processes using sand pack column.

KEYWORDS— Oil recovery, Bacteria, Biosurfactants, Lipases, Sand pack column

INTRODUCTION

Oil existence in waters and land was considered to be undesirable, both because of its quantity and in the wrong place [1]. Product of oil processing industry had potentially polluted the environment and caused damage or disturb the living things. Waste produced by oil processing was in form of hydrocarbon compound. Hydrocarbon waste formed from leaks on transportation pipe and raw oil storage tank damage, both were capable of causing pollution [2].

The effective and efficient method to handle oil waste pollution was to elevate oil recovery, by surfactant administration. Surfactant (*surface active agent*) was amphipathic molecule consisted of hydrophobic and hydrophilic groups. Chemical surfactant usage possibly caused environmental problem of its resistant properties and high toxicity when it accumulated on natural ecosystem [3], so various environmental-friendly surfactant as alternative means started to be developed. One of it was by using microorganism which known as biosurfactant. Biosurfactant capable of declining surface tension, increasing solubility of hydrophobic compound contained in oil, and extending facilitation for microbe on hydrocarbon degradation [4].

Lipases were one material also able to elevate oil recovery. Lipases addition could increase oil recovery effectiveness because it properties in degrading oil hydrocarbon compound. In addition, hydrocarbonoclastic microbe also produced certain compound namely biosurfactant [5]. Because of that, formula addition of potential microbe consortium from lipase-producing bacterial group was necessary on furthering oil recovery effectiveness [6].

Oil recovery effectiveness affected by biosurfactant and lipase types used to dissolve the oil. Effectiveness in oil waste recovery process using biosurfactant and lipase rarely applied before. For that, it required to test oil waste mobility using sand pack column. Sand pack column method was one of many kind method used to determine oil recovery capability.

Biosurfactant-producing bacterial used in this study were *Acinetobacter sp.* P2(1), *Bacillus subtilis* 3Kp, *Pseudomonas putida* T1-8, and *Micrococcus sp.* L II 61, while lipase-producing bacterial that used were *Actinobacillus sp.* and *Micrococcus sp.* L II 61. Treatments consisted of 8 combination; *Acinetobacter* sp. P2(1) - *Actinobacillus* sp., *Bacillus subtilis* 3Kp - *Actinobacillus* sp., *Pseudomonas putida* T1-8 - *Actinobacillus* sp., *Micrococcus* sp. L II 61 - *Actinobacillus* sp., *Acinetobacter* sp. P2(1) - *Micrococcus* sp. L II 61, *Bacillus subtilis* 3Kp - *Micrococcus* sp. L II 61, *Pseudomonas putida* T1-8 - *Micrococcus* sp. L II 61 and *Micrococcus* sp. L II 61 biosurfactant and lipase, with synthetic surfactant (Tween-20) as positive control.

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METHODS

This study conducted in Microbiology Laboratory of Biology Department, Faculty of Science and Technology, Airlangga University at January – May 2014.

A. *Lipase and Biosurfactant Production Medium Preparation*

Lipase production medium used is 93 ml Nutrient Broth mixed with 2 ml cooking oil, sterilized in 500 ml bottle. Biosurfactant production medium used Synthetic Mineral Water (SMW) Medium, made by dissolving 3 g $(NH_4)_2SO_4$, 0.2 g $MgSO_4.7H_2O$, 10 g NaCl, 0.01 g $CaCl_2$, 0.001 g $MnSO_4.H_2O$, 0.001 g H3BO3, 0.001 g $ZnSO_4.7H_2O$, 0.001 g $CuSO_4.5H_2$, O0.005 g $CoCl_2.6H_2O$, and 0.001 g $Na_2M_oO_4.2H_2O$ into 980 mL aquadest and 20 mL diluted molasse in aquadest (1:1). The solution homogenized using magnetic stirer and its pH neutralized by adding 10% NaOH or 5% HCl. Solution then put into 500 mL tube. Sterilized SMW and molasse medium is macronutrient solution. While micronuterient solution made from 1 g KH_2PO_4, 1 g K_2HPO_4 and 1 g $FeSO_4.7H_2O$ in 50 mL aquadest stock, which then sterilized. Both nutrient solutions mixed in aseptic condition.

B. *Biosurfactant Supernatant and Lipases Characteristic Test*

Bacterial cells in culture incubated for 4 days separated from medium containing biosurfantant with 9000 rpm, 4ºC centrifuge for 15 minutes [7]. Before recovering oil, potential and characteristic of biosurfactant bacterial supernatant was tested, because each bacterial have different ability on oil recovery, which would be affected solubility percentage. The abilities of supernatant acquired tested by measuring surface tension and emulsification activity.

Lipase characteristic tested by calculated lipolytic activity if crude enzyme, measured quantitatively using modified [8] method. Lipolytic activity was determined using spectrophotometric method in *p*-nitrofenil palmitat (*p*-NPP) substrate.

C. *Oil Recovery Test using Sand Pack Column Method*

In mobilization test using sand pack column, initially sand filtered using size 40 mess, then it washed with acids (5% HCl) one times and shaken down to homogenized it. Then it was rinsed using aquadest for 3 times, dried by putting it on the oven of 50°C temperature, and weighted for 30 g. Sand mixed with 10 ml crude oil to saturate it. Sand mixture put into sand pack column, which beneath covered with milipore filter membrane (Whatman No.1). Biosurfantant and lipase (1:1) formulated solution of 10 ml added to it. Negative control used was aquadest and positive control used was Tween-20 synthetic surfactant. Formulated solution of biosurfactant and lipase flooded in glass tube was left in for 12-24 hours. Dissolved oil measured by extracting disintegrating oil from sand pack column using gravimetric method. N-hexane solvent added to the resulting solution, the solvent evaporated for ± 20 minutes in n-hexane boiling point of 60-70^0 C. Resulting oil of evaporation weighted.

RESULTS AND DISCUSSION

A. Supernatant characteristic of Biosurfactant and Lipase

1) Surface Tension

Table 1 showed that *Bacillus subtilis* 3Kp bacteria was able to decline surface tension as much as 9.14 dyne/cm. According to [9], surface tension decreased up to ≥10 dyne caused by production factor, of not optimum *Bacillus subtilis* 3Kp incubation time. *Bacillus subtilis* was surfactant producing microbe from subtilicine type.

Acinetobacter sp. P2(1) and *Pseudomonas putida* T1(8) bacteria were able to decrease surface tension by 16.89 dyne/cm and 10,56 dyne/cm. *Acinetobacter sp.* P2(1) and *Pseudomonas putida* T1(8) biosurfactant bacteria were able to descend surface tension as much as ≥10 dyne/cm. Both product of biosurfactant had small molecule weight, it indicated by high amount of surface tension decline [10].

Micrococcus sp. L II 61 reduced surface tension up to 17.82 dyne/cm. These results informed that *Micrococcus sp.* L II 61 also capable of declining surface tension by ≥10 dyne/cm. *Micrococcus sp.* L II 61 had the highest result in reducing surface tension.

Table 1. Surface Tension of 4 Types Supernatant Containing Biosurfactant

Treatment	Surface Tension (dyne/cm)	Surface Tension Reduction (dyne/ml)
Molasse without microbe	59.70 ± 1.35	-
Molasse + *Bacillus subtilis* 3Kp	50.56 ± 0.64	9.14
Molasse + *Acinetobacter* sp. P2(1)	42.81 ± 1.55	16.89
Molasse + *Pseudomonas putida* T1(8)	49.14 ± 1.85	10.56
Molasse + *Micrococcus* sp. L II 61	41.88 ± 1.60	17.82

2)

3) Emulsification Activity

Emulsification activity (EA) test also important in representing biosurfanctant production by *Bacillus subtilis* 3Kp, *Pseudomonas putida* T1-8, *Micrococcus sp.* L II 61 and *Acinetobacter sp.* P2(1). Emulsion stability (%) was show the strength of biosurfactant produced by microbes in emulsifying hydrocarbon [11].

Table 2 shows that *Bacillus subtilis* 3Kp able to emulsify oil as much as 18.85% with low stability percentage of 4.05%, while *Acinetobacter sp.* P2(1) and *Micrococcus sp.* L II 61 able to emulsify raw oil by 24.94% and 63.97%, both condition were relatively stable after 24 hours, by 20.88% and 61,50% with low stability percentage of 4.48% and 2.47%. Biosurfactant of *Pseudomonas putida* T1(8) was able to emulsify raw oil by 40.64% with high stability percentage of 34.4%. From this results, it concluded that the four bacterias capable of producing bioemulsifiers compound with low stability percentage, except *Pseudomonas putida* T1(8). But the highest emulsification activity acquired from *Micrococcus* sp. L II 61.

Table 2. Emulsification Activity for 1 hour and 24 hours (%) of supernatant containing biosurfactant towards crude oil

Treatment	Average Emultification Activity (%)		Reduction of Emultification Activity (%)
	1 hour	24 hours	
Tween-20 control	47.14 ± 13.9	46.39 ± 13.04	0.75
Bacillus subtilis 3Kp	18.85 ± 2.90	14.38 ± 4.05	4.05
Acinetobacter sp. P2(1)	24.94 ± 2.92	20.88 ± 3.47	4.48
Pseudomonas putida T1(8)	40.64 ± 10.38	6.25 ± 0.58	34.4
Micrococcus sp. L II 61	63.97 ± 5.31	61.50 ± 2.28	2.47

4) Lipolytic Activity Test

Lipase catalyzed triglycerides that hydrolyzed into diglycerides, monoglycerides, glycerols, and fatty acids. Triglycerides were split into fatty acid and glycerol by lipase is called lipolytic activity. Lipolytic activity of lipase crude enzyme measured quantitatively using *p*-nitrofenil palmitat (*p*-NPP) as testing substrate.

Table 3 showed that *Micrococcus* sp. L II 61 had the higher activity amount by 11.137 (U/ml), while *Actinobacillus* sp. had the lower activity average of 7.553 (U/ml). Both bacterias have lipolytic ability and can be used in oil recovery test.

Table 3. Lipolytic activity and dry weight of Lipase crude enzyme raw product

Bacterial Lipase	Average Activity (U/ml)	Dry Weight (g)
Micrococcus sp. L II 61	11.137 ± 0.566	0.762
Actinobacillus sp.	7.553 ± 2.99	0.435

B. Extraction Result of Biosurfactant and Critical Micelle Concentration (CMC) Determination

After biosurfactant produced and supernatant acquired, supernatant then extracted to obtain biosurfactant crude product. Biosurfactant dry weight acquired from 60% ammonium sulfate precipitation which obtained from *Bacillus subtilis* 3Kp, *Pseudomonas putida* T1-8, *Micrococcus* sp. L II 61 and *Acinetobacter sp.* presented on Table 4. After crude product obtained, CMC from *Acinetobacter* sp. P2(1), *Pseudomonas putida* T1-8, *Bacillus subtilis* 3Kp and *Micrococcus* sp. L II 61 biosurfactant was determined.

Table 4. Dry weight of biosurfactant crude product extracted from 100 mL supernatan

Biosurfaktant Bacteria	Dry weight of crude product (g)	CMC (g/L)	Description
Bacillus subtilis 3Kp	6.111	16	≤CMC
Acinetobacter sp. P2(1)	4.494	5	≤CMC
Pseudomonas putida T1-8	3.782	3.75	≥CMC
Micrococcus sp. L II 61	4.017	4.73	≤CMC

Results was show that the four bacteria studied produced biosurfactant on different level. It had various emulsification activity and surface tension. From Table 4, it had known that the crude product of biosurfactant of *Acinetobacter sp.* P2(1) was as much as 5 g/L, while *Pseudomonas putida* T1-8 and *Micrococcus* sp. L II 61was 3,75 g/L and 4,73 g/L respectively, and *Bacillus subtilis* 3Kp crude product weighted 16 g/L.

Previous study [12] stated on the same concentration with CMC (=CMC), biosurfactant would be formed more mycelles. This statement supported by high percentage of oil recovery effectiveness from *Acinetobacter sp.* P2(1) and *Pseudomonas putida* T1(8) on the same concentration with CMC (=CMC); respectively 48,62% and 13,81%. But those oil recovery effectiveness acquired from biosurfactant crude product application, while this study applied supernatant directly on oil recovery using sand pack column, where biosurfactant combined with lipase not always affected by the concentartion (< CMC, = CMC, > CMC), towards oil recovery and percentage of crude oil solubility. Biosurfactant optimization might be conducted by increasing production or incubation duration, so the product acquired CMC (=CMC) or CMC (≥CMC).

Determination of CMC from the four biosurfactant crude products; *Bacillus subtilis* 3Kp, *Pseudomonas putida* T1-8, *Micrococcus sp.* L II 61, and *Acinetobacter sp.* P2(1), purposed to understand the different characteristic of the four bacterias used. According to [9], surface tension, CMC, and emulsification stability were features and characteristics biosurfactant

product depending on substrate and bacteria type used. Efficiency and effectiveness of the four biosurfactants determined from CMC and surface tension [13].

A biosurfactant expressed effectively when it had 1-2000 mg/L [14]. Based on CMC and surface tension, biosurfactant crude product of *Bacillus subtilis* 3Kp, *Pseudomonas putida* T1-8, *Micrococcus sp.* L II 61 and *Acinetobacter sp.* P2(1) categorized as less efficient, because the total CMC values >2000 mg/L. This was possibly caused by the form of biosurfactant that was crude product and still yet to be purified.

C. Supernatant Combination of Biosurfactant and Lipase Effectiveness Test in Oil Recovery using Sand Pack Column

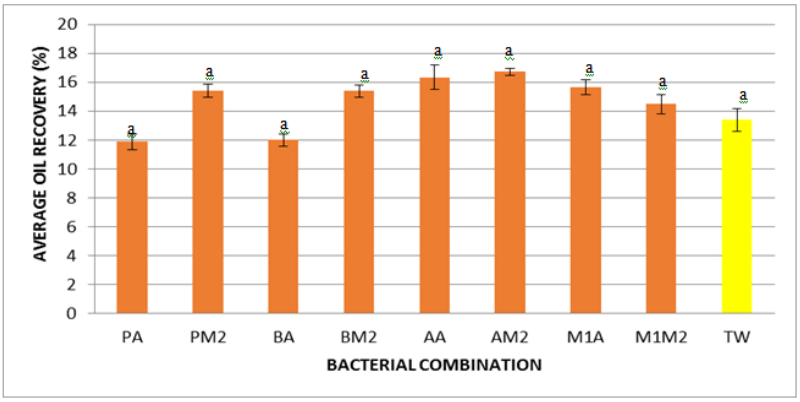

Fig. 1 Graphic of biosurfactant and crude enzyme combination effect on oil recovery percentage

Description:
TW : Tween 20 (positive control)
AA : *Acinetobacter* sp. P2(1) biosurfactant and *Actinobacillus sp.* lipase
BA : *Bacillus subtilis* 3Kp biosurfactant and *Actinobacillus sp.* lipase
PA : *Pseudomonas putida* T1(8) biosurfactant *Actinobacillus sp.* lipase
M1A : *Micrococcus* sp. L II 61 biosurfactant and *Actinobacillus sp.* lipase
AM2 : *Acinetobacter* sp. P2(1) biosurfactant and *Micrococcus sp.* L II 61 lipase
BM2 : *Bacillus subtilis* 3Kp biosurfactant and *Micrococcus sp.* L II 61 lipase
PM2 : *Pseudomonas putida* T1(8) biosurfactant and *Micrococcus sp.* L II 61 lipase
M1M2 : *Micrococcus* sp. L II 61 biosurfactant and *Micrococcus sp.* L II 61 lipase

The result of oil recovery from various treatments conducted on the study presented on Fig 1. Oil recovery percentage from the eight biosurfactant and lipase combination analyzed statistically. Results of Kolmogorov-Smirnov test showed that data collected have normal distribution. Then data examined using Levene test and resulting in homogenous data type. With one-way ANOVA, data of the eight treatment combination of biosurfactant and lipase showed no significant effect on oil recovery using sand pack column method with significance degree (α) > 5 % (0,05).

Based on Fig 1, the eight combination treatment had not effected greatly on oil recovery using sand pack column method. The effectiveness in oil recovery of the eight combinations was all comparable to that of synthetic surfactant (Tween-20). Oil recovery using aquadest (negative control) reached to 1.62%, it was because a substance can be dissolved into solvent if both possess the same polarity; such as polar substance would be dissolved into polar solvent and not dissolved into non-polar solvent. Oil (lipids)was a non-polar compound, while aquadest was polar solvent. So the oil can't be dissolved into aquadest [15].

Results of oil recovery in positive control (Tween-20) obtained as much as 13.40%. The highest result from bacteria treatment acquired from combination of *Acinetobacter sp.* P2(1) biosurfactant and *Micrococcus* sp. L II 61crude enzyme, with percentage of 16,73%.

Overall, all treatment conducted was able to enhance oil recovery, as evidenced by the resulting oil recovery percentage comparable to that of synthetic surfactant (Tween-20). The ability in removing oil caused by lipase crude enzyme works on oil (lipid)-water interfacial and degrades well oil (lipid) component on crude oil until it can be dissolved into water phase.

But the oil recovery of biosurfactant and lipase combination using sand pack column method did not result in high percertage compared to solubilization test using agitation method. From [16] study, *Bacillus subtilis* 3Kp biosurfactant combined with *Actinobacillus* sp. Were able to dissolve oil sludge as much as 40,671% on the same concentration of CMC, and from [7] study, *Acinetobacter sp.* P2(1) biosurfactant dissolvde oil sludge by 40,93% on the same concentration of CMC.

Declines of oil recovery percentage on sand pack column method possibly caused by biosurfactant negative interaction factor with sand (soil) substrate. Sand or soil type used in the study affect greatly in releasing hydrocarbon bond with biosurfactant and crude oil. In addition, other factors also influence oil recovery percentage on sand pack column.

Although resulting oil recovery percentage was not quite high, but the effectiveness of *Bacillus subtilis* 3Kp, *Pseudomonas putida* T1-8, *Micrococcus* sp. L II 61, *Acinetobacter* sp. P2(1) biosurfactant combined with *Actinobacillus* sp. and *Micrococcus* sp. L II 61 crude enzyme supernatant almost on the same level with synthetic surfactant (Tween-20). This

indicated that biosurfactant combination with lipase crude enzyme was potentially able to substitute synthetic surfactant usage in crude oil waste processing.

Results obtained in this study came from one times flushing or recover with 24 hours exposure time. Soil polluted with spilled oil will produce more optimum results by multiple flushing. But this theory was still yet to be proven; if oil recovery with multiple flushing would be resulted in increasing, decreasing, or constant percentage. It was interesting to reveal how oil recovery percentage resulted with application of multiple flushing on the four biosurfactant and two lipase types studied.

CONCLUSION

1. The four types of biosurfactant had different characters. *Bacillus subtilis* 3Kp was able to decline surface tension to 9.14 dyne/cm and emulsify oil as much as 18.85%. *Acinetobacter* sp. P2(1) was able to decrease surface tension up to 16.89 dyne/cm and emulsify oil by 24.94%. *Pseudomonas putida* T1(8) and *Micrococcus* sp. L II 61 was able to decline surface tension by 10,56 dyne/cm and 17.82 dyne/cm respectively, and also emulsified oil as much as 63.97% and 40.64%.
2. Both lipase types had different characters. *Micrococcus* sp. L II 61 and *Actinobacillus sp.* had lipolytic ability and possibly applied on oil recovery, respectively 11.137 (U/ml) and 7.553 (U/ml).
3. Combination of *Bacillus subtilis* 3Kp, *Pseudomonas putida* T1-8, *Micrococcus* sp. L II 61 biosurfactant with *Micrococcus* sp. L II 61 and *Acinetobacter* sp. P2(1) lipase had effected and resulted comparable to those of synthetic surfactant (Tween-20) on oil recovery using sand pack column method.

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